LIII. NOTES ON SOME FURTHER EXPERIMENTS ON THE CLOTTING OF CASEINOGEN SOLUTIONS.

BY SAMUEL BARNETT SCHRYVER.

From the Research Institute of the Cancer Hospital.

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Some months ago, I communicated to the Royal Society the results of a series of experiments on the clotting of caseinogen solutions [1913]. It was there shown that caseinogen, when freshly precipitated from diluted skimmed-milk under certain specified precautions, is an unstable substance which on treatment with water at 37°, or with 0.1% acetic acid at room temperature, is readily converted into another product or series of products (metacaseinogens), which differ from the original substance in that they are very much less soluble in lime water, and consist therefore apparently of smaller aggregates of lower molecular weight. When a half-saturated solution of lime water is saturated with the natural caseinogen by rotating the mixture for several hours in a thermostat at 20°, an acid milky solution is obtained after centrifugalisation and filtration, which readily clots without the addition of a soluble calcium salt in the presence of rennin; it will also clot in the absence of rennin when gently warmed (to 25°) in the presence of soluble calcium salts when the latter exist in the mixture within certain definite limits of concentration. In the presence of milk-serum, peptones, amino-acids etc., a clot can be produced from the above-mentioned calcium caseinogenate solution only in the presence of both soluble calcium salts and rennin.

In the paper quoted it was shown, furthermore, that the casein of the clot produced from calcium caseinogenate by rennin alone is about half as soluble in lime water as the natural caseinogen, and appears to possess, therefore, approximately half the molecular weight, and corresponds in properties with the metacaseinogens produced from natural caseinogen by the action of water.

Similar conclusions as to the molecular weight of casein, and its relations to caseinogen have been arrived at recently by Lucius van Slyke and A. W. Bosworth [1913], who have attacked the problem from a quite different

standpoint, and by other experimental methods, and in a still more recent paper by Bosworth [1913] a method is described for the production of a clot from caseinogen solutions by rennin alone, which does not differ materially from the method described by me¹.

Bosworth also came to the same conclusion as I did, that the production of casein from caseinogen is not an ordinary proteoclastic reaction, although it may be the result of a hydrolytic cleavage. I have ventured to suggest that the two substances bear a relationship to one another similar to that of metaphosphates to pyrophosphates. Bosworth and I are also in accord with reference to the identity, or at any rate the approximate identity, of the empirical chemical compositions of caseinogen and casein.

Accurate stoichiometrical relationships between casein and caseinogen were not to be attained by the method of experiment adopted by me, owing to the existence of an adsorption equilibrium when lime water is treated with excess of various caseinogen and casein preparations, a point which has been the subject of numerous experiments, which are briefly mentioned in my previous paper [1913, p. 466].

The casein prepared from the clot differs in one important respect from metacaseinogen produced by the action of water at 37° or by 0.1% acetic acid at room temperature from caseinogen, for whereas the latter, on solution in excess of caustic alkali and reprecipitation from this solution with acetic acid under certain defined conditions is reconverted into a caseinogen of relatively high solubility in lime water, which readily yields clots on treatment either with calcium chloride or rennin, casein obtained by means of rennin cannot be converted into caseinogen under these conditions; on dissolving in alkali and reprecipitation, a product of only about half the solubility of caseinogen in half-saturated lime water is obtained. The solution in lime water furthermore does not clot on addition of rennin, and yields, on mixture with solutions of calcium salts within certain limits of concentration, an immediate precipitate. This precipitate is readily distinguishable from the clot produced under similar circumstances from caseinogenate solutions, which only forms when the temperature is raised above that of the temperature of solution.

Further experiments have been directed towards ascertaining the reason of this difference between the casein of the clot and the metacaseinogen

¹ Throughout my papers, I have consistently referred to the preparations which have not been clotted as caseinogen, whereas the preparations obtained from the clot are described as casein. In this respect, I have kept to the English method of nomenclature. Van Slyke and Bosworth adopt the method of nomenclature usual on the continent, describing the products as casein and paracasein, respectively.

produced by the action of water alone on caseinogen. These experiments are not yet complete, but I have been tempted to communicate them in their present form, owing to the fact that they have been subjected to a temporary interruption due to my change of laboratories.

CLOTTING OF CASEINOGEN SOLUTIONS BY MEANS OF PANCREATIN.

Investigations have been carried out with the object of comparing the process of clot production by means of other ferments. The results obtained with pancreatin indicate that its action can be readily distinguished from that of pepsin (or rennin). When half-saturated lime water is saturated with natural caseinogen, the solution thus obtained in the manner described [Schryver, 1913, p. 462] readily clots on the addition of small amounts of pepsin solution (1%). *No soluble calcium salt need be added.* A clot under similar conditions cannot be produced by a pancreatin solution, although the latter will clot milk. A soluble calcium salt appears, therefore, to be necessary when pancreatin is employed for clot-production [compare Mellanby, 1912]. A clot was also prepared from an artificial milk by pancreatin in the following way: 5 c.c. of calcium caseinogenate solution were mixed with an equal bulk of milk-serum containing calcium chloride in the concentration of N/25. Under these conditions, as has already been shown, no clot is produced. If to this mixture are added a few drops of 1% pancreatin solution, and the whole is incubated, a clot is produced similar in appearance to that produced by pepsin under like conditions.

The clot produced from artificial milk has the same properties as that produced from the natural milk, but differs in certain important respects from the clot produced by pepsin (or rennin) preparations.

The general method employed for examining the casein from the clots was as follows. The serum was poured off from the clot, which was then thrown on calico, and squeezed. It was then ground in a mortar with 0.1% acetic acid, washed with water, dilute alcohol in increasingly graded strengths, absolute alcohol and ether, and then air-dried. The fine powder thus produced was then heated for 5 minutes with absolute alcohol to destroy the enzyme, the hot alcohol was filtered off by means of a Buchner funnel and the powder then washed with alcohol and ether, and again air-dried. The powder was then transferred to a mortar, ground with water into a paste, and N sodium hydroxide solution was added with continual grinding, until nearly all dissolved, and the solution was alkaline to phenolphthalein. It was then diluted and filtered through paper-pulp on to a Buchner funnel. Dilute

acetic acid was then carefully added with constant stirring until precipitation was complete, the supernatant liquid was rapidly poured off, and the precipitate washed by decantation or on a filter with ice-cold water, alcohol in graded strengths up to absolute alcohol, and finally with ether, and it was then air-dried.

Sometimes the above process was modified in various details, e.g. the clot after washing with water was first dissolved in alkali and reprecipitated, and the product after drying in the usual manner treated with hot alcohol. In all cases, however, a similar product was obtained. When the clots produced by pepsin and pancreatin were compared, the same procedure was always employed. The clots produced by pepsin alone from calcium caseinogenate solution, or by the combined action of the ferment and calcium chloride and ferment in the presence of milk-serum, yielded caseins with the same properties.

Now whereas the original caseinogen preparations had a solubility in half-saturated lime water of about 35¹, and after solution in alkalis and reprecipitation a solubility of about 30, the solubility of the casein preparation from the pepsin clot was about 14–17 and that of the pancreatin clot about 6–8. Unlike metacaseinogen, therefore, caseins are not converted into the more soluble products by solution in alkali and reprecipitation with acids, even when all precautions for preventing subsequent change have been taken. Furthermore, a saturated solution of caseinogen in half-saturated lime water is opaque like milk, whereas that of pepsin-casein is opalescent, and that of pancreatin-casein is generally water-clear. The two latter yield immediate precipitates and not clots on treatment with calcium chloride and do not clot on treatment with rennin. There appears, therefore, to be a difference in the molecular weights of the caseins produced by pepsin and pancreatin, both of which are considerably lower than that of caseinogen, that of pepsin-casein being about one-half and that of pancreatin-casein about one-quarter of that of caseinogen. Again, attention must be called to the fact that exact stoichiometrical relationships are not to be expected from the method of experiment employed.

THE DIFFERENCES BETWEEN METACASEINOGENS AND CASEINS.

As already repeatedly stated, the metacaseinogens differ from the caseins in that the latter are not reconverted into caseinogen on solution in alkali

¹ I.e. 5 c.c. of the solution after Kjeldahlisation required 35 c.c. N/10 sulphuric acid to neutralise the ammonia produced (see former paper).

and reprecipitation with acids, whereas the former are. In spite of the large number of experiments which have been carried out, I have not so far succeeded in producing from a casein a solution which clots on treatment with rennin.

Investigations have, therefore, been carried out with the object of ascertaining the reason of the differences of behaviour between these substances, which in other respects have similar properties.

Up to the present, no entirely satisfactory proof as to the actual nature of the difference has been found, but certain facts have been recently discovered which afford some indication.

It seems not improbable that the ferment forms a combination with its substrate, from which it cannot afterwards be separated, and that the properties of the latter are then so altered that it can no longer form the big aggregates of the character of caseinogen.

Experiments were therefore carried out with the object of ascertaining the properties of the substance produced, when ferments were allowed to act on caseinogen at low and high temperatures, i.e., under conditions under which the proteoclastic action of the ferment is excluded.

Pepsin was allowed to act on calcium caseinogenate for 15 minutes at 0°, the conditions of experiment being otherwise the same as those employed in clot production. No clot formed under these conditions. The free caseinogen was then precipitated with ice-cold dilute acetic acid and a little sodium chloride¹, and rapidly washed several times with ice-cold water, graded strengths of alcohol, absolute alcohol and ether. These operations were carried out as rapidly as possible. The powder after air drying was then heated for 5 minutes with boiling alcohol, dissolved in excess of alkali, from the solution in which the casein(ogen) was obtained in the usual way. Although the product thus obtained possessed about the same solubility in half-saturated lime water as ordinary caseinogen which had been dissolved in alkali and reprecipitated, the solution obtained from caseinogen which had been treated with the pepsin differed from that got from normal caseinogen (treated in a similar way but without pepsin), in that the former did not clot in the presence of rennin, and yielded an immediate precipitate, and not a clot, when treated with calcium chloride solution of the requisite concentration.

The mere precipitation therefore, of caseinogen in the presence of a ferment under conditions which exclude enzyme action, causes appreciable alterations in its properties. When one considers the important part that surface

¹ The precipitate does not readily separate in ice-cold solutions without this addition.

actions play in such processes, this result is not altogether surprising. The above result was obtained several times. A similar experiment was also carried out in which pancreatin was used instead of pepsin, the conditions being the same as those described above. The product in this case had a solubility of only about 8 in half-saturated lime water, and was identical in properties with the casein obtained from a pancreatin clot. It may be remarked here that pancreatin produces the clot in the highly acid solution.

A large number of experiments were also carried out with the object of ascertaining the action of the heated ferment on clot formation. The results obtained up till now are not, however, quite conclusive, and this is due to the varying properties of the different pepsin preparations. Rohonyi [1913] has recently succeeded in preparing definite compounds of proteins with proteoclastic ferments, and has shown that such compounds can be obtained when ferment solutions are employed, the proteoclastic activity of which has been destroyed by heat. If the conception is true that the caseins differ from metacaseinogens in the fact that the former are combinations with the ferments, then, in the light of Rohonyi's recent work, it is conceivable that caseins should be formed by the action of heated enzymes. In carrying out the experiments devised for testing this theory precisely the same difficulties have been encountered as those described by Rohonyi, due to the differences of characters of the various enzyme preparations. This investigator has shown that it is possible to prepare compounds of pepsin and proteins by the employment of solutions of the ferment which have been heated for 15 minutes at 100° *provided that no precipitate is formed during the heating*. Preparations having this property appear to be rare. I have myself not succeeded in obtaining such. I have, however, obtained a preparation which after heating to 80° for some time was still capable of actively clotting calcium caseinogenate solutions. Prolonged heating of the ferment solution in quartz tubes at this temperature produced a precipitate, and the solution gradually became inactive.

It is very probable that experiments of this nature will only succeed when a ferment preparation relatively free from extraneous proteins is obtained. Further work in this direction is contemplated.

THE NATURE OF "NATURAL CASEINOGEN."

L. van Slyke and Bosworth have called attention to the fact that various commercial preparations of caseinogen contain large quantities of acid calcium salt, due apparently to the precipitation of the product with insufficient quantities of acid. A criticism might therefore be directed against the "natural caseinogen" described by me, that such consisted of a mixture of free acid and acid calcium salt. I therefore subjected my preparation to examination with a view of ascertaining its nature, and comparing it with a calcium-free preparation prepared according to the method of van Slyke and Bosworth. The latter was found to have a low solubility in half-saturated calcium hydroxide, as it had stood some time in contact with hydrochloric acid during the process of filtration. After solution in alkali, and reprecipitation with acid, with the precautions already repeatedly described, it had a solubility of about 26, i.e., about the solubility of my own caseinogen preparations which have been "purified" by solution in alkali and reprecipitation by acid¹.

The solubilities of my "natural caseinogen" and of the caseinogen preparations prepared according to van Slyke and Bosworth (both the preparation directly precipitated with hydrochloric acid and that redissolved in alkali and reprecipitated by acetic acid with precautions for preventing change) in warm 10% sodium chloride were determined. Van Slyke and Bosworth show that under these conditions the calcium salts readily dissolve, whereas the free acids do not. The preparations were mixed with 10 times their weight of the saline solution and maintained at 37° for about 1 hour with continual shaking. The mixtures were afterwards centrifuged, and the nitrogen in the clear filtered supernatant liquid was determined by Kjeldahl's process.

The saline extract from all preparations contained about the same small amount of nitrogen, viz. 2 or 3 mgrms. in 5 c.c. Only minute quantities of the preparations were soluble in saline, and my "natural caseinogen" is therefore a free acid and not a calcium salt. As a control, an acid calcium salt was prepared by precipitation of sodium caseinogenate solution by calcium chloride [Schryver, 1913, p. 469]. The precipitate was washed with dilute alcohol till chlorine free, and obtained dry after alcohol and ether washing in the usual way. On treating this with 10 times its weight of 10% sodium chloride at 37°, it rapidly dissolved, leaving only a very small residue, and yielding a thick syrupy solution.

¹ Preparations thus "purified" have never as high a solubility in lime water as "natural caseinogen."

CONCLUSIONS.

1. Casein produced from a pepsin (or rennin) clot differs from meta-caseinogen, a product produced by the action of water at 37° on caseinogen, in that it cannot be converted by solution in alkali and reprecipitation into a more soluble product which dissolves in calcium hydroxide to yield clottable solutions. The statement made in my earlier paper that casein produced by pepsin is an aggregate of about half the size of that of caseinogen is confirmed.

2. The action of pancreatin in clot-production differs from that of pepsin, in that it produces clots only in the presence of soluble calcium salts. It produces these clots, however, under conditions under which calcium salts alone fail, e.g., in the presence of milk-serum. The casein from pancreatin clots also differs from that of pepsin clots. The former have only about half the solubility in half-saturated lime water of the latter. Such lime water solutions are usually water-clear.

3. Certain experiments are quoted, which indicate that the difference between metacaseinogens and caseins is due to the fact that the latter are combinations of the enzyme with the protein. The evidence produced both by myself and independently by van Slyke and Bosworth and by Bosworth alone, indicate that the action of the ferment is not an ordinary proteoclastic action. It is conceivable that if the enzyme contains both haptophoric and zymophoric groups, the former only take part in clot formation.

4. Evidence is brought to show that the "natural caseinogen," the preparation of which is described in my earlier paper, is not a calcium salt.

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